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Structure of the major carrot allergen Dau c 1

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Dau c 1 is a major allergen of carrot (*Daucus carota*) which displays IgE cross-reactivity with the homologous major birch-pollen allergen Bet v 1. The crystal structure of Dau c 1 has been determined to a resolution of 2.7 Å, revealing tight dimers. The structure of Dau c 1 is similar to those of the major allergens from celery, Api g 1, and birch pollen, Bet v 1. Electron density has been observed in the hydrophobic cavity of each monomer and has been modelled with polyethylene glycol oligomers of varying length. Comparison of the surface topology and physicochemical properties of Dau c 1 and Bet v 1 revealed that they may have some, but not all, epitopes in common. This is in agreement with the observation that the majority of carrot-allergic patients have Bet v 1 cross-reactive IgE antibodies, whereas others have Dau c 1-specific IgE antibodies which do not recognize Bet v 1.

1. Introduction

Carrot (*Daucus carota*) allergy belongs to the group of pollen-related food allergies, with a prevalence of 25% among food allergies (Ballmer-Weber *et al.*, 2001). In Europe, carrot allergy is generally based on primary sensitization to birch pollen and only in rare cases has carrot sensitization been observed without concomitant pollen allergies (Fernández-Rivas *et al.*, 2004; Moneo *et al.*, 1999). Homologous allergenic proteins present in birch pollen as well as in the edible parts of carrots provide the molecular basis for the observed clinical cross-reactivity to both allergen sources. Cross-reactivity occurs when the IgE antibodies originally produced in response to Bet v 1 sensitization recognize analogous epitopes which are present on the surface of the plant proteins (Valenta & Kraft, 1996). Consequently, re-exposure to pollen or homologous plant allergens will provoke an allergic reaction in already sensitized individuals (Fritsch *et al.*, 1997; Bohle *et al.*, 2003). To date, several allergenic proteins have been identified in carrot: Dau c 1, Dau c 4 (profilin), an isoflavone reductase, nonspecific lipid-transfer protein (nsLTP) and cross-reactive carbohydrates (CCDs). Of those, Dau c 1 represents the major allergen which induces the production of specific IgE antibodies in 98% of carrot-allergic patients (Ballmer-Weber *et al.*, 2001; Hoffmann-Sommergruber *et al.*, 1999).

Dau c 1 and other plant proteins homologous to the major birch-pollen allergen Bet v 1 belong to group 10 of the pathogenesis-related proteins (PR-10), which are intracellular proteins that are constitutively expressed in certain plant tissues during development. Moreover, these proteins are up-regulated by microbial attack, fungal elicitors, wounding or other physical or chemical stress (Hoffmann-Sommergruber & Radauer, 2004). Their function as plant steroid carriers has

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been proposed (Marković-Housley *et al.*, 2003). Dau c 1 consists of a small number of isoforms composed of 154 (or 153) amino-acid residues: Dau c 1.0103 (accession No. Z81361, EMBL GenBank database), Dau c 1.0104 (accession No. Z81362, EMBL GenBank database) and Dau c 1.0201 (AF456481). Dau c 1.0103 and Dau c 1.0104 (Hoffmann-Sommergruber *et al.*, 1999) share 97% identity with each other (Hoffmann-Sommergruber *et al.*, 1999; Ballmer-Weber *et al.*, 2005), 50% sequence identity with Dau c 1.0201 and 37–38% sequence identity with Bet v 1 (Ballmer-Weber *et al.*, 2005). A recombinant Dau c 1.0201 isoform has been shown to display different IgE-binding ability in sera from carrot-allergic patients, thus suggesting epitope diversity between Dau c 1.0104, Dau c 1.0103, Dau c 1.0201 and Bet v 1 (Ballmer-Weber *et al.*, 2005). In general, Bet v 1-homologous proteins in plant foods are regarded as thermolabile proteins that undergo rapid loss of their three-dimensional structure, which in turn reduces the allergenic potential of these proteins. However, T-cell epitopes can survive and trigger T-cell reactivity and thus can influence the T-cell-mediated late-phase skin reactions in patients with atopic dermatitis (Bohle *et al.*, 2006). Bet v 1 homologues have been identified in the past in apple, cherry and celery and their relevance for component-resolved diagnosis of food allergy has been corroborated (Vieths *et al.*, 1994; Scheurer *et al.*, 1997; Hoffmann-Sommergruber *et al.*, 1999). The beneficial effect of birch-pollen immunotherapy (IT) on concomitant plant food allergies is controversial, as has been shown for apple allergy (Bolhaar *et al.*, 2004; Kinaciyan *et al.*, 2007). Bolhaar and coworkers performed birch-pollen IT in a group of birch-pollen-allergic and apple-allergic patients and showed that pollen-allergic symptoms as well as apple-allergic symptoms were reduced. This beneficial effect was observed over 2 years following the treatment. In contrast, Kinaciyan and coworkers observed no effects on apple-allergic symptoms in patients when performing birch-pollen-based immunotherapy (Kinaciyan *et al.*, 2007). Recently, promising results have been obtained by studying the effects of vaccination with hypoallergenic Bet v 1 derivatives on the immunological and clinical responses to food allergens in patients with pollen-related oral allergy syndrome (OAS; Niederberger *et al.*, 2007). Additional strategies should be considered if birch-pollen IT fails, such as replacing Bet v 1 by the specific plant food allergen.

Reliable structures of the plant food allergens are needed as a molecular basis for the identification of surface areas important for cross-reactivity as well as areas representing potential additional new epitopes. To date, three-dimensional structures are available for the major birch-pollen allergen Bet v 1.2801 (Gajhede *et al.*, 1996) and its natural hypoallergenic isoform Bet v 1l (Marković-Housley *et al.*, 2003) and the major allergens from cherry (Pru av 1; Neudecker *et al.*, 2001) and celery (Api g 1; Schirmer *et al.*, 2005), as well as for PR-10 proteins from yellow lupin in the apo form (Biesiadka *et al.*, 2002; Pasternak *et al.*, 2005) and in complex with physiologically relevant ligands such as *trans*-zeatin (Fernandes *et al.*, 2008) and a synthetic cytokinin (Fernandes *et al.*, 2009). In addition, the structure of the cytokinin-specific binding protein VrCSBP in complex with zeatin has been reported and showed a fold similar to those of PR-10 proteins despite the low sequence identity (<20%). However, classification of VrCSBP into the PR-10 family has not yet been experimentally confirmed; moreover, according to a recent classification by Radauer *et al.* (2008) it belongs to another subfamily.

The fold characteristic of all these structures consists of a seven-stranded β-sheet and three α-helices which enclose a large hydrophobic cavity. The aim of the present study was to identify the three-dimensional structure of a relevant food allergen and to compare it with already known structures of the Bet v 1 family in order to provide deeper insights into the structural determinants relevant to cross-reactivity with clinical implications and to address questions of component-resolved diagnosis and IT.

2. Material and methods

2.1. Crystallization and data collection

Recombinant Dau c 1.0103 (accession No. Z81361, EMBL GenBank database) was expressed by *Escherichia coli* M15 harbouring the plasmid pDS 56/Dau c 1 and was purified by nickel-chelate affinity chromatography (Hoffmann-Sommergruber *et al.*, 1999). Cubic crystals of Dau c 1 were grown by the hanging-drop vapour-diffusion method at 293 K as follows: equal volumes (1.5 µl) of protein dissolved in 20 m*M* Tris–HCl buffer pH 8.35 (8–10 mg ml^{-1}) and of precipitant solution (2% PEG 400, 0.1 *M* sodium citrate, 2.0 *M* ammonium sulfate) were mixed and equilibrated against 0.7 ml of the latter solution. Crystals suitable for X-ray analysis grew within 1–2 weeks. Prior to data collection, crystals were soaked briefly in a cryoprotective solution (Paratone-N) and the diffraction data were collected at the SLS (Swiss Light Source) using a MAR CCD detector. All measurements were performed at 100 K.

The images were indexed and integrated using the program *MOSFLM* (Leslie, 1992). The crystals belonged to the cubic space group $P4_132$, with unit-cell parameters $a = b = c = 189.9$ Å. There are four monomers of Dau c 1 per asymmetric unit, resulting in a solvent content of 72.3% (V_M = 4.4 Å^3 Da^{-1}; Matthews, 1968). The data were reduced and scaled using programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994; Table 1).

2.2. Structure solution and refinement

The structure was solved by the molecular-replacement method using the crystal structure of the homologous major celery allergen Api g 1 (PDB code 2bk0; Schirmer *et al.*, 2005) as the search model. All side chains and solvent molecules beyond C^β were removed from the search model (poly-Ala). Rotation and translation functions were calculated using the program *MOLREP* (Vagin & Teplyakov, 1997), yielding orientations and positions for four protomers with a correlation coefficient of 0.22 (30–4 Å). Rigid-body refinement resulted in R and R_{free} factors of 50.6% and 48.5%, respec-

Table 1
Crystallographic data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
X-ray source	SLS-PX
Wavelength (Å)	0.978
Temperature (K)	100
Detector type	MAR CCD
Space group	$P4_132$
Unit-cell parameters (Å)	$a = b = c = 189.9$
No. of molecules in ASU	4
Resolution range (Å)	77.6–2.7 (2.85–2.7)
Total No. of observations	112198 (15618)
No. of unique observations	31520 (4482)
Completeness (%)	96.8 (96.5)
Multiplicity	3.6 (3.5)
$\langle I/\sigma(I)\rangle$	13.0 (3.1)
$R_{\rm merge}$† (%)	8.9 (39.6)
Refinement statistics	
Resolution range (Å)	30–2.7 (2.77–2.7)
Total No. of reflections	29881 (2154)
R factor/$R_{\rm free}$‡ (%)	22.3/23.7 (37.8/40.3)
Protein atoms	4464
Water molecules	16
Chloride anions	16
PEG atoms	130
Average B factor (Å^2)	
Protein atoms	26
Water molecules	26
Chloride anions	43
PEG atoms	39
R.m.s.d. from ideal values	
Bond lengths (Å)	0.01
Bond angles (°)	1.22
Ramachandran plot (%)	
Favoured region	97.3
Allowed region	2.7
Disallowed region	0.0

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity in the ith data set and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl over all measurements of $I(hkl)$. ‡ R factor is the conventional R factor and $R_{\rm free}$ is the R factor calculated with 5% of the data that were not used in refinement.

tively. The initial electron density was improved by NCS averaging (*DM*; Cowtan, 1994). After modelling of the side chains, the model was refined with NCS restraints, TLS parameters (four bodies) and individual B factors using *REFMAC* (Winn *et al.*, 2001). Rounds of manual rebuilding using the program *O* (Jones *et al.*, 1991) and refinement yielded the final model (Table 1). The progress of the refinement was monitored by the decrease in the free R factor while minimizing the divergence between R and $R_{\rm free}$. The model quality was checked with *PROCHECK* (Laskowski *et al.*, 1993).

Fig. 1 was produced with the program *DINO* (http://www.dino3d.org) and Figs. 2(*a*), 3 and 4 were produced with the program *SPDBV* (Guex & Peitsch, 1997). The solvent-accessible surface areas include contributions from the main chain and side chains of the residues involved.

3. Results and discussion

3.1. Crystal structure of Dau c 1

The crystal structure of the recombinant major carrot allergen Dau c 1 was determined to a resolution of 2.7 Å (Table 1). Molecular replacement using the structure of Api g 1 (PDB code 2bk0; Schirmer *et al.*, 2005) as a search model provided the orientation and location of the four Dau c 1 copies in the asymmetric unit. The protomers are arranged into two virtually identical dimers *AB* and *CD* (Fig. 1). The initial electron-density map was improved by cyclic real-space averaging (*DM*). Refinement with strong NCS restraints yielded a model consisting of two virtually identical dimers with R and $R_{\rm free}$ values of 22.3% and 23.7%, respectively (Table 1). The final model (Table 1, Fig. 1) comprises protein chains *A*–*D* (Ala3–Asn154), 16 water molecules, 16 chloride ions and 130 polyethylene glycol (PEG) atoms. The overall fold of Dau c 1 (Figs. 1 and 2*a*) consists of a seven-stranded antiparallel β-sheet (β1–β7) which together with a long C-terminal helix (α3) and two additional short

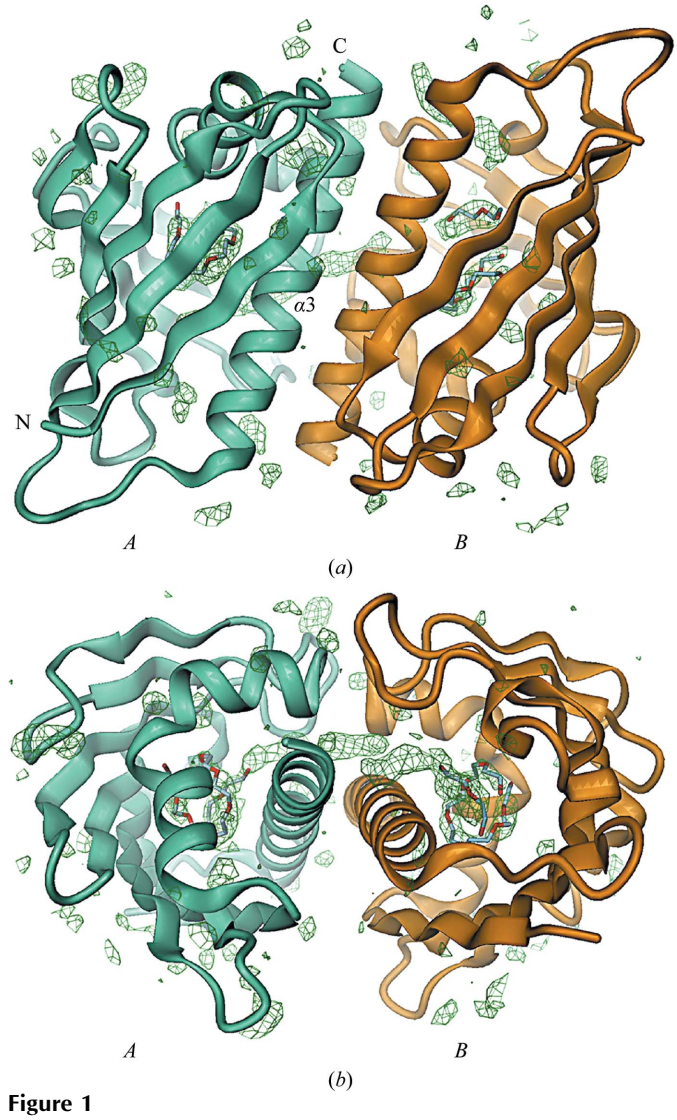

Figure 1
Schematic view of the Dau c 1 dimer with residual density ($F_{\rm o} - F_{\rm c}$ map) contoured at 2.8σ. The four monomers, *A*–*D*, associate into two virtually identical dimers *AB* (gold and green) and *CD* (not shown). The additional electron density observed in the hydrophobic cavity of each monomer was tentatively modelled with molecules of polyethylene glycol. The view in (*b*) is rotated around the horizontal axis by 90° with respect to the view in (*a*).

helices (α1 and α2) confines a large hydrophobic cavity, a fold typical for all members of the Bet v 1-related protein family.

After refinement of the protein structure, residual density (Fig. 1) remained in the central hydrophobic cavity of each molecule. Density which obeyed the local symmetry was tentatively modelled with a short and a long (spiral-like) fragment of PEG. Two other short PEG fragments were positioned close to the contact point of chain *B* with its crystallographic symmetry mate. The source of the PEG molecules could be the polydisperse PEG 400 sample used for crystallization. All four monomers of the asymmetric unit are virtually identical and superimpose with an r.m.s.d. in the range 0.25–0.28 Å for all C^{α} positions. Therefore, the following discussion is restricted to chain *A*.

In the crystal structure, Dau c 1 exists as a dimer which is held together by isologous contacts formed by residues of helix α3 (Fig. 1). In contrast, in solution recombinant Dau c 1 is present as a monomer, as shown by gel-filtration chromatography (B. Maderegger, personal communication). The dimer interface was determined to be 709 $Å^2$, which is below the consensus cutoff value (~850 $Å^2$) which distinguishes between dimers formed by crystal contacts (<850 $Å^2$) and true physiological dimers in solution (Ponstingl *et al.*, 2000). Thus, it appears that Dau c 1 exists as a monomer in solution and that the dimerization is driven by crystallization forces.

Table 2
Superposition of the carbon C^{α} chains of crystallographic models of Dau c 1 (chain *A*) and various members of the Bet v 1 family.

Values in parentheses are the amino-acid sequence identities.

	R.m.s. deviation (Å)/No. of C^{α} atoms						
Protein	Api g 1	Bet v 1	Bet v 1l	LlPR-10.2A	LlPR-10.2B	LlPR-10.2B	VrCSBP
PDB code	2bk0	1bv1	1fm4	1xdf	2qim	3e85	2flh
Ligand†	None	None	Deoxycholate	None	*Trans*-zeatin	DPU‡	*Trans*-zeatin
Dau c 1 2vjg PEG§	0.71/152 (81)	1.15/143 (38)	1.18/140 (35)	1.35/111 (37)	1.34/120 (37)	1.23/123 (37)	1.50/97 (18)

† Protein structure in complex with ligand. ‡ *N*,*N*′-Diphenylurea. § Polyethylene glycol molecules of various lengths.

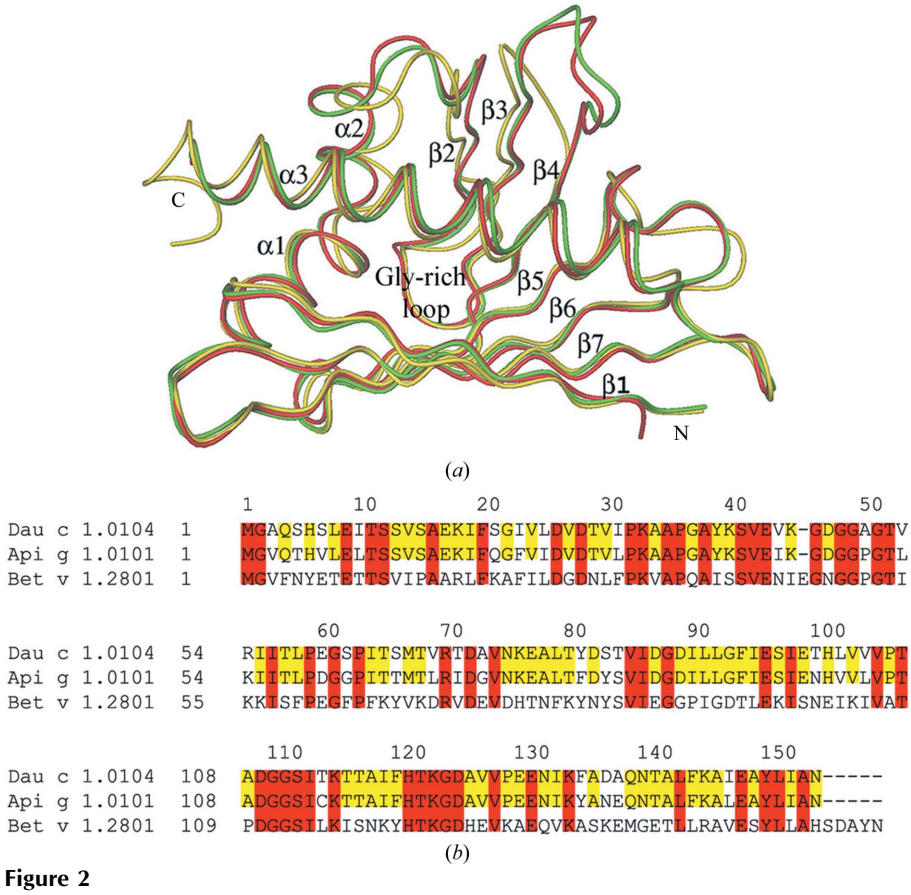

Figure 2
(*a*) Ribbon representation of the superimposed backbones of the crystal structures of Dau c 1 (red), Api g 1 (green) and Bet v 1 (yellow). The secondary-structure elements and the highly conserved Gly-rich loop are labelled. (*b*) Sequence alignment of Dau c 1.0103 with Api g 1.0101 (81% identity, coloured yellow) and with Bet v 1 (residues that are fully conserved in all three proteins are printed on a red background).

3.2. Structural comparison of Dau c 1 with other members of the Bet v 1 family

Dau c 1 and all known PR-10 proteins have a common overall fold consisting of a seven-stranded antiparallel β-sheet (β1–β7) and three α-helices which enclose a large hydrophobic cavity suitable for binding of hydrophobic ligands. The structural comparison of Dau c 1 in complex with PEG molecules with known structures of other unliganded and liganded proteins of the Bet v 1 family (Table 2) may give a hint as to whether ligand binding induces noticeable structural changes despite the conservation of the overall fold, as discussed below.

The superposition of the C^{α}-atom pairs of Dau c 1 and its unliganded homologue from celery Api g 1 (81% sequence identity; Fig. 2*a*) resulted in an r.m.s. deviation of 0.7 Å for 152 C^{α} atoms (Table 2). This indicates moderate but significant structural differences which may in part arise from the bound PEG molecules. Major structural changes are seen for the residues of the β2 strand and neighbouring loops (r.m.s.d. of 1.0 Å for 16 C^{α} atoms) and in the loops joining strands β3 and β4 (r.m.s.d. of 1.4 Å for six C^{α} atoms).

The comparison of Dau c 1 with unliganded birch pollen Bet v 1 (38% sequence identity) and the hypoallergenic isoform Bet v 1l in complex with deoxycholate (35% sequence identity) revealed similar structural changes (r.m.s.d.s of 1.15 and 1.18 Å, respectively; Table 2) which were more significant than those observed when comparing Dau c 1 with Api g 1. The highest discrepancies observed in Bet v 1 (Fig. 2*a*, Table 2) and Bet v 1l (Table 2) compared with Dau c 1 involved residues 23–45 (r.m.s.d. of 4.2 Å for 23 C^{α}atoms), which belong to helix α2 and strand β2 (plus neighbouring loops), as well as the loops between β3 and β4 (r.m.s.d. of 2.1 Å for six C^{α} atoms) and β5 and β6 (r.m.s.d. of 2.8 Å for six C^{α} atoms). In the Bet v 1l–deoxycholate complex only one additional loop joining strand β7 and helix α3 was significantly changed (r.m.s.d. of 2.4 Å for ten C^{α} atoms), probably because the residues of this loop interact with the outer deoxycholate molecule (Marković-Housley *et al.*, 2003).

The Dau c 1 structure was also compared with those of yellow lupin PR-10 proteins (37% sequence identity) which are available in the apo form (LlPR-10.2A; Pasternak *et al.*, 2005) and in complex with ligands [LlPR-10.2B in complex with *trans*-zeatin (Fernandes *et al.*, 2008) and LlPR-10.2B in complex with synthetic cytokinin (Fernandes *et al.*, 2009)] (Table 2). The structural variations between Dau c 1 and yellow lupin proteins are rather high (average r.m.s.d. of 1.3 Å) and are similar for the unliganded and liganded forms (Table 2). Therefore, it is difficult to evaluate the effect of ligand binding on the observed structural changes in the compared studies. The comparison of Dau c 1 with a distant cytokinin-specific binding protein from mung bean (VrCSBP; 18% sequence identity) in complex with zeatin (Pasternak *et al.*, 2006) revealed the highest structural divergence (r.m.s.d. of 1.50 Å for only 97 C^{α} atoms) with regard to other compared proteins. This is not surprising in view of the low sequence identity and suggests that there are some structural features that are only characteristic of VrCSBP. The major structural differences are seen in helices α2 and α3 and in the loops joining strands β3 to β4, β5 to β6 and β6 to β7. Interestingly, the quaternary structures of Dau c 1 and VrCSBP are quite similar. Both asymmetric units contain four monomers (*A*, *B*, *C* and *D*) which form two identical dimers *AB* and *CD*. In Dau c 1 the dimers are held together by contacts formed by residues of the α3 helix, whereas in VrCSBP the monomers interact through residues of their α1/α2 region.

3.3. Epitopes of Dau c 1 that are cross-reactive with Api g 1 and Bet v 1

The cross-reactivity between the major pollen allergen Bet v 1 and related allergens from carrot (Dau c 1), celery (Api g 1) and other fruits and vegetables has been well established (Ebner *et al.*, 1995; Hoffmann-Sommergruber *et al.*, 1999; Breiteneder *et al.*, 1995). The cross-reactivity occurs when the structurally similar epitopes present on plant food proteins recognize and bind to the IgE antibodies specific to Bet v 1. Cross-inhibition experiments using the immunoblot method have shown that recombinant rApi g 1 and rBet v 1 were able to abolish binding of patient IgE antibodies to rDau c 1.0103, thus suggesting that Api g 1 and Bet v 1 contain all the epitopes present on Dau c 1.0103 (Hoffmann-Sommergruber *et al.*, 1999).

This is not surprising, particularly for Api g 1.0101, which has surface properties that are highly similar to those of Dau c 1.0103 (Figs. 3 and 4). The sequence variation between Dau c 1 and Api g 1 comprises 30 amino acids (Fig. 2*b*) which are equally confined to both the protein surface and protein interior. In view of the highly similar surface properties (Fig. 3 and Fig. 4), it is conceivable that Dau c 1 and Api g 1 share the same cross-reactive epitopes with Bet v 1. Indeed, the three cross-reactive epitopes which have been proposed to account for pollen–celery cross-reactivity (Schirmer *et al.*, 2005) are also conserved in Dau c 1.

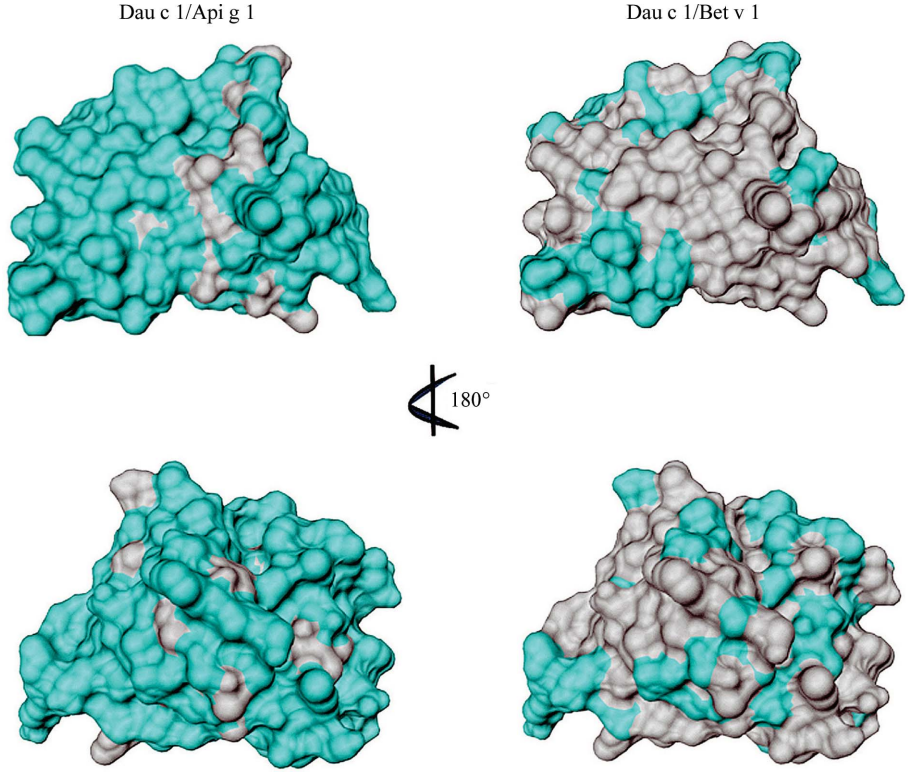

Figure 3
Solvent-accessible surfaces of Dau c 1, Api g 1 and Bet v 1 viewed as in Fig. 2(*a*) and the rear view with molecules rotated by 180° around the *y* axis (bottom row). Cyan colour denotes residues that are conserved between Dau c 1 and Api g 1 (left) and between Dau c 1 and Bet v 1 (right). Non-identical residues are shown in grey.

Comparison of Dau c 1 with Bet v 1 shows that although they have the same fold there are some differences in surface topology defined by the side chains and in the distribution of charged, aromatic, polar and apolar residues (Figs. 3 and 4) which are determinants of the allergen–antibody interaction. Despite these differences, carrot allergy is highly associated with a sensitization to birch pollen (Wüthrich *et al.*, 1990; Hoffmann-Sommergruber *et al.*, 1999). In fact, in a double-blind placebo-controlled food challenges (DBPCFFCs) study with 20 patients with histories of allergic reactions to carrot (Ballmer-Weber *et al.*, 2001), all DBPCFC-positive patients were also sensitized to birch pollen. A follow-up study also showed that binding of IgE from the sera of these patients to Dau c 1 was inhibited by rBet v 1 (Ballmer-Weber *et al.*, 2001). This again points out that despite the relatively low sequence conservation between Bet v 1 and Dau c 1 (38%) the two allergens must share common epitopes. However, for a small subset of DBPCFC-positive patients the binding of IgE was not inhibited or was weakly inhibited by Bet v 1 or birch-pollen extracts, indicating that Dau c 1 also contains unique B-cell epitopes that differ from those of Bet v 1. Indeed, inspection of Fig. 3 suggests that several unique epitopes can be accommodated within the grey surface area that comprises exposed residues that are non-identical in Dau c 1 and Bet v 1. Thus, it is likely that secondary sensitization to discrete epitopes on the major allergen Dau c 1 can occur in a subgroup of carrot-allergic patients independently of a primary sensitization to birch-pollen allergens (Ballmer-Weber *et al.*, 2001). In this context, the study of Moneo *et al.* (1999) supports this proposal by demonstrating that some carrot-allergic patients had significant levels of IgE antibodies specific to carrot but lacked the IgEs specific to Bet v 1.

4. Concluding remarks

The analysis of the crystal structure of the major carrot allergen Dau c 1.0103, which is related to the major birch-pollen allergen Bet v 1, enabled insight into the molecular basis of cross-reactivity between the inhalant birch-pollen allergen and the plant food allergen. The highly homologous celery allergen Api g 1 largely shares the same three-dimensional structure and surface properties with Dau c 1 and both proteins crystallize as dimers. In contrast, Bet v 1 and Dau c 1 have common surface areas as well as areas that are significantly different. These distinct areas of the carrot allergen may bear the Dau c 1-specific epitopes which can lead to carrot

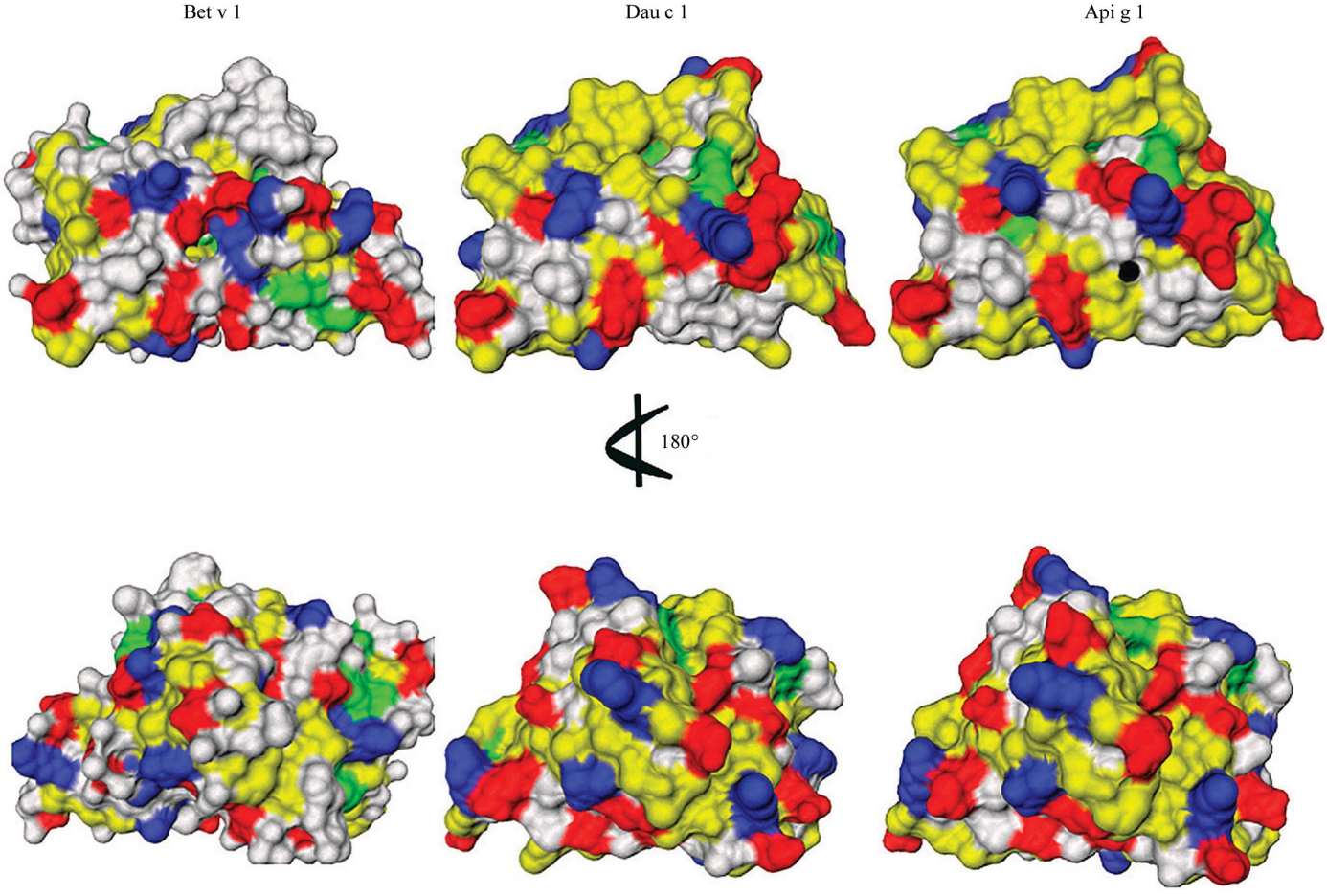

Figure 4
The molecular surfaces of Dau c 1, Api g 1 and Bet v 1 viewed as in Fig. 2(*a*) and rotated by 180° around the vertical axis (bottom row). The surface is colour-coded according to residue type, with red, blue, yellow, green and grey representing acidic, basic, apolar, aromatic and polar residues (other than basic and acidic residues), respectively.

sensitization without concomitant birch-pollen allergy. Furthermore, detailed epitope analysis of the individual Bet v 1-related allergens will contribute to the identification of common epitopes as targets for immunotherapy of pollen-related food allergies.

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